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Flavone *C*-Glycosides from Leaves of *Oxalis triangularis*

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The flavone *C*-glycosides luteolin 6-*C*-(2″-*O*-β-xylopyranosyl-β-glucopyranoside) (**1**), apigenin 6-*C*-(2″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**2**), apigenin 6-*C*-(2″-*O*-β-xylopyranosyl-β-glucopyranoside) (**3**), apigenin 6-*C*-(2″-*O*-(6‴-(*E*)-caffeoylglucoside)-β-glucopyranoside) (**4**), and apigenin 6-*C*-(2″-*O*-(6‴-(*E*)-*p*-coumaroylglucoside)-β-glucopyranoside) (**5**) have been isolated from the purple leaves of *Oxalis triangularis*. Compound **4** is new, while **5** has previously been isolated from *Cucumis sativus* after treatment with silicon and infection with *Sphaerotheca fuliginea*. Signal duplication in the NMR spectra of **2**, **4**, and **5** revealed the presence of rotameric conformers, created by rotational hindrance at the *C*(sp^3) −*C*(sp^2) glucosyl−flavone linkage in these flavone *C*-glycosides.

KEYWORDS: Purple shamrock; *Oxalis triangularis*; leaves; acylated flavone *C*-glycosides; rotameric conformers; 2D NMR

INTRODUCTION

Oxalis triangularis A. St.-Hil. (purple shamrock or purple clover) in Oxalidaceae is an edible perennial plant which is easily cultivated. The leaves are especially appreciated because of their sour and exotic taste. The family Oxalidaceae comprises more than 900 species belonging to seven genera, namely, *Averrhoa*, *Dapania*, *Biophytum*, *Eichleria*, *Hypseocharis*, *Oxalis*, and *Sarcotheca*. The genus *Oxalis* includes 800 of the species in Oxalidaceae (*1*). A few species in the family belonging to the genera *Averrhoa*, *Biophytum*, and *Oxalis* (altogether eight) have previously been analyzed with respect to their flavonoid content. Alcalde-Eon et al. (*2*) identified malvidin 3-acetylglucoside-5-glucoside in addition to the 3,5-diglucosides of peonidin, petunidin, and malvidin and the 3-glucosides of peonidin, delphinidin, petunidin, and malvidin in colored tubers of oca (*Oxalis tuberosa* Mol.) var. Isla Oca. The anthocyanins from the leaves of *O. triangularis* were recently identified as seven acylated and nonacylated malvidin glycosides (*3*, *4*). Cyanidin 3-glucoside has been identified as the main pigment in callus cultures of *Oxalis reclinata* Jacq. (*5*), and the *C*-glycosylflavones apigenin 8-*C*-glucoside (vitexin), apigenin 6-*C*-glucoside (isovitexin), and vitexin 2″-glucosylglucoside have been isolated from *Oxalis corniculata* (*6*). Other *C*-glycosylflavones such as isovitexin 2″-glucosylglucoside has been isolated from wood sorrel (*Oxalis acetosella*) (*7*).

O. triangularis has intensely purple leaves with a monomeric anthocyanin content of 195 mg/100 g on a malvidin 3,5-diglucoside basis which make them a potential source for natural colorants (*3*). It is known that flavone *C*-glycosides as copigments have effects on the color and stability of anthocyanins (*8*−*11*). In this study we have undertaken the isolation and structure determination of flavone *C*-glycosides from the leaves of *O. triangularis*.

EXPERIMENTAL PROCEDURES

Isolation. Purple shamrock was cultivated in Bergen. A voucher specimen has been deposited in Bergen Herbarium, University of Bergen (accession number H/505). The leaves of *O. triangularis* (100 g) were cut into pieces and extracted twice with 1 L of 0.5% TFA in MeOH at 4 °C. The filtered extract was concentrated under reduced pressure, purified by partition three times against an equal volume of EtOAc, and then subjected to Amberlite XAD-7 column chromatography (*12*, *13*). The flavonoids were further purified on a 100 × 5 cm Sephadex LH-20 column using MeOH−H_2O−TFA (19.8:80:0.2, v/v) as an eluent. The flow rate was 2.5 mL/min. Using this solvent composition, 2200 mL was eluted. Then the mobile phase composition was changed to MeOH−H_2O−TFA (39.8:60:0.2, v/v), and a further 2745 mL was eluted prior to the elution of pigment **3** (260 mL), followed by the fraction containing pigment **4** (280 mL). The flavonoids were then finally purified by preparative HPLC according to previously published procedures (*14*). Altogether 14 mg of **4**, 15 mg of **5**, 46 mg of **3**, 2 mg of **1**, and 3 mg of **2** were isolated.

Analytical HPLC. Analytical HPLC was performed with a 250 × 4 mm i.d., 5 μm, ODS-Hypersil column (ThermoQuest, Cheshire, U.K.) using the solvents HCOOH−H_2O (1:18) (A) and HCOOH−H_2O−MeOH (1:8:10) (B). Gradient 1 consisted of a linear gradient from 10% B to 100% B for 23 min, 100% B for the next 5 min, and a linear gradient from 100% B to 10% B for 1 min. The flow rate was 0.75 mL/min, and aliquots of 15 μL were injected.

Spectroscopy. UV/vis absorption spectra were recorded on-line during HPLC analysis with a Hewlett-Packard HP1050 multidiode array detector over the wavelength range 240−600 nm in steps of 2 nm. The NMR experiments were obtained at 600.13 and 150.92 MHz for ^1H and ^{13}C, respectively, on a Bruker DRX-600 instrument equipped with a multinuclear inverse probe for all but the ^{13}C 1D CAPT experiment, which was performed at 100.61 MHz on a Bruker DMX-400 instrument equipped with an BBO probe. Sample temperatures were stabilized at 25 °C. The deuteriomethyl ^{13}C signal and the residual ^1H signal of the solvent (CF_3CO_2D−CD_3OD, 5:95, v/v) were used as

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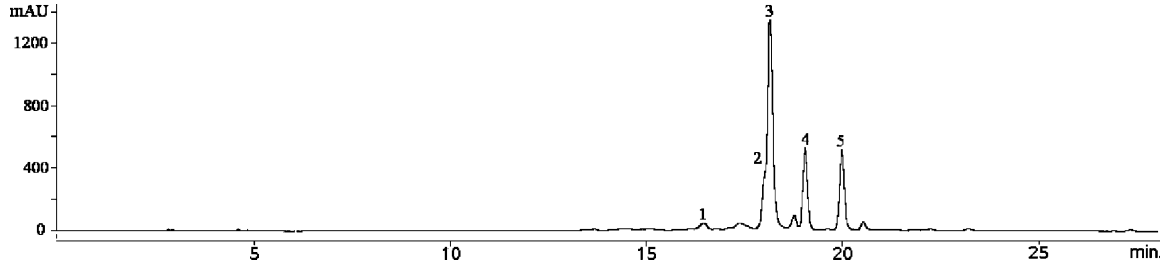

Figure 1. HPLC profile of the flavones in the crude extract of the leaves of *O. triangularis* recorded at 360 ± 20 nm.

secondary references (δ 49.0 and δ 3.40 from TMS, respectively). A compensated attached proton test (CAPT) experiment was performed with 20864 transients. The spectral width was 20161 Hz. The one-bond proton−carbon shift correlations were established using phase-sensitive gradient-selected heteronuclear single quantum coherence (HSQC). The experiment was optimized for a one-bond proton−carbon coupling constant of 145 Hz. A total of 256 FIDs were recorded in t_1 and 2K data points in t_2, and 132 transients were collected for each t_1 increment. The spectral widths were 18110 Hz in f_1 and 3501 Hz in f_2. The proton−carbon shift correlations by long-range coupling were established using a heteronuclear multiple-bond correlation (HMBC) experiment. A total of 256 FIDs were recorded in t_1 and 2K data points in t_2, and 210 transients were collected for each t_1 increment. The spectral widths were 24147 Hz in f_1 and 3501 Hz in f_2. The one-bond proton−proton shift correlations were established using phase-sensitive gradient-selected double-quantum-filtered correlation spectroscopy (DQF-COSY) with solvent suppression. The experiment was optimized for a proton−proton coupling constant of 7.5 Hz. A total of 256 FIDs were recorded in t_1 and 4K data points in t_2, and 30 transients were collected for each t_1 increment. The spectral width was 1407 Hz. The total correlations between the protons belonging to each sugar unit were established by total correlation spectroscopy (TOCSY) experiments (Bax and Davis, 1985). A total of 256 FIDs were recorded in t_1 and 4K data points in t_2, and 32 transients were collected for each t_1 increment. The spectral width was 1407 Hz. A nuclear Overhauser and exchange spectroscopy (NOESY) experiment was performed with 256 FIDs recorded in t_1 and 2K data points in t_2, and 32 transients were collected for each t_1 increment. The spectral width was 3501 Hz.

Low-resolution mass spectrometric data were achieved by a LCMS system (Waters 2690 HPLC system connected to a Micromass LCZ mass spectrometer) with electrospray ionization in positive mode (ESP+). The following ion optics were used: capillary 3 kV, cone 30 and 60 V, and extractor 7 V. The source block temperature was 120 °C, and the desolvation temperature was 150 °C. The electrospray probe flow was adjusted to 100 μL/min. Continuous mass spectra were recorded over the range *m*/*z* 150−800 with a scan time of 1 s and an interscan delay of 0.1 s. High-resolution mass spectra: The flavones were dissolved in methanol−1% formic acid (1:1, v/v). Approximately 3 μL of this solution (final concentration ca. 20 pmol/L) was added to a gold-coated nanospray glass capillary (Protana, Odense, Denmark). The tip of the capillary was placed orthogonally in front of the entrance hole of a quadrupole time-of-flight (QTOF 2) mass spectrometer (Micromass, Manchester, Great Britain) equipped with a nanospray ion source, and a voltage of approximately 1000 V was applied. The isotopic composition of the sample was determined in the accurate mass mode using stachyose and cyclodextran (Glc7) ($[M + H]^+ = 667.0994$; 1135.3776 Da) as internal reference compounds.

Pigment Identification. Compound **1**: R_t (HPLC) 17.72 min, $\lambda_{UV_{max1}}$ 350 nm, $\lambda_{UV_{max2}}$ 272 nm, ESI low-resolution MS (M^+) *m*/*z* 581. Compound **2**: R_t (HPLC) 19.70 min, $\lambda_{UV_{max1}}$ 339 nm, $\lambda_{UV_{max2}}$ 272 nm, ESI low-resolution MS (M^+) *m*/*z* 579. Compound **3**: R_t (HPLC) 19.88 min, $\lambda_{UV_{max1}}$ 340 nm, $\lambda_{UV_{max2}}$ 272 nm, ESI low-resolution MS (M^+) *m*/*z* 565. Compound **4**: R_t (HPLC) 21.08 min, $\lambda_{UV_{max1}}$ 332 nm, $\lambda_{UV_{max2}}$ 274 nm, ESI high-resolution MS (M^+) *m*/*z* 757.199 (calcd 757.198) corresponding to $C_{36}H_{37}O_{18}$. ^{1}H NMR (CD_3OD at 25 °C. glc = glucopyranosyl, m = multiplet, s = singlet, d = dublet, dd = double dublet, t = triplet, br = broad. Two chemical shift values are given for rotameric conformers): *Apigenin* δ 6.35 s, δ 6.56 s (H-3); δ 6.48 s (H-8); δ 7.69 'd', 8.9 (H-2′,6′); δ 6.90 m (H-3′,5′); 6-β-*C-glc* δ 5.05 d, 9.9 (H-1″); δ 4.38 m (H-2″); δ 3.79 t, 9.0 (H-3″); δ 3.61 dd, 9.4, 9.0 (H-4″); δ 3.50 m (H-5″); δ 3.97 d br, 12.0 (H-6A″); δ 3.83 dd, 12.0, 5.4 (H-6B″); 2″-β-*O-glc* δ 4.51 s br (H-1‴); δ 3.25 m (H-2‴); δ 3.37 m (H-3‴); δ 3.35 m (H-4‴); δ 3.28 m (H-5‴); δ 4.23 d br, 12.0 (H-6A‴); δ 3.97 d br, 12.0 (H-6B‴); 6‴-*caffeoyl* δ 6.71 m (H-2⁗); δ 6.81 m (H-5⁗); δ 6.90 m (H-6⁗); δ 6.01 d, 15.9 (H-α); δ 7.19 d, 15.9 (H-β). Compound **5**: R_t (HPLC) 22.25 min, $\lambda_{UV_{max1}}$ 320 nm, $\lambda_{UV_{max2}}$ 276 nm, ESI low-resolution MS (M^+) *m*/*z* 741.

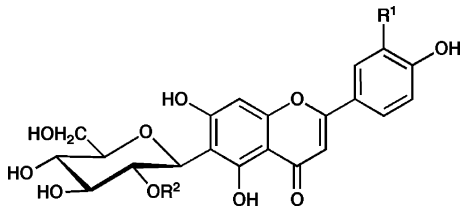

Figure 2. Structures of flavone *C*-glycosides **1**–**5** from *O. triangularis*: (**1**) R^1 = OH, R^2 = xylosyl, (**2**) R^1 = H, R^2 = rhamnosyl, (**3**) R^1 = H, R^2 = xylosyl, (**4**) R^1 = H, R^2 = 6‴-(*E*)-caffeoyl-*O*-β-glucosyl, (**5**) R^1 = H, R^2 = 6‴-(*E*)-*p*-coumaroyl-*O*-β-glucosyl.

RESULTS AND DISCUSSION

The HPLC profile of the acidified, methanolic extract of the leaves of *O. triangularis* detected at 360 nm showed three major and several minor flavonoids (**Figure 1**). The aqueous concentrate of the acidified methanolic extract of the leaves of *O. triangularis* was purified by partition against ethyl acetate followed by Amberlite XAD-7 column chromatography. The flavonoids in the purified extract were fractionated by Sephadex LH-20 column chromatography. Individual pigments **1**−**5** (for their structures, see **Figure 2**) were separated by preparative HPLC.

Identification. The UV/vis spectrum of **4** recorded on-line during HPLC analysis showed absorption bands around 332 nm with increased absorption around 320 nm in accordance with a flavone or flavonol acylated with a cinnamic acid (*15*). The downfield part of the 1D ^{1}H NMR spectrum of **4** showed a 4H AA′XX′ system at δ 7.69 (semi-d, J = 8.9 Hz, H-2′/6′) and δ 6.90 (H-3′/5′), two 1H singlets at δ 6.48 (H-8) and δ 6.35 (H-3), a 3H AMX system at δ 6.90 (H-6⁗), δ 6.81 (H-5⁗), and δ 6.71 (H-2⁗), and a 2H AX system at δ 7.19 (J = 15.9 Hz, H-β) and δ 6.01 (J = 15.9 Hz, H-α) in accordance with a 6-*C*-substituted apigenin glycoside acylated with (*E*)-caffeic acid. The sugar region showed the presence of two sugar units. The anomeric coupling constant (J = 9.9 Hz) and the 12 ^{13}C resonances in the sugar region of the ^{13}C CAPT spectrum of **4** were in accordance with two β-glucopyranose units (*16*). All the ^{1}H sugar resonances were assigned by the 2D ^{1}H−^{1}H COSY and TOCSY spectra, and the corresponding ^{13}C resonances were then assigned by the 2D ^{1}H−^{13}C HSQC experiment. The 24 ^{13}C resonances in the 1D ^{13}C CAPT spectrum of **4** belonging to the aglycone and the acyl moiety were assigned (**Table 1**) by the cross-peaks in the 2D ^{1}H−^{13}C HMBC and HSQC spectra,

Table 1. ^{13}C NMR Spectroscopic Data for **1**–**5** at 25 °C[a]

	1[b,c]	**3**[b]	**3**[d]	**2**[b,c]	**4**[b]	**5**[d]
			Aglycone			
2	nd	165.96	163.57	166.1	166.13	163.58
3	103.8	103.73	102.84	104.0	103.82	102.77
						102.63
4	nd	183.88	182.02	184.2	184.00	182.11
						181.72
5	nd	158.63	161.26	nd	162.57	159.79
6	nd	108.96	108.09	109.4	109.19	108.03
						107.98
7	nd	164.92	163.57	164.7	164.92	163.58
8	94.7	94.82	93.37	95.8	95.25	93.85
			93.12	94.6		92.82
9	nd	158.63	156.45	158.8	158.62	156.39
10	nd	105.07	103.40	105.4	105.10	103.44
			103.34			103.01
1′	nd	122.95	121.18	123.1	123.10	121.34
2′	114.1	129.34	128.57	129.5	129.50	128.38
3′	nd	117.00	116.09	117.1	116.79	115.82
4′	nd	162.63	161.26	162.8	162.57	161.10
5′	116.8	117.00	116.09	117.1	116.79	115.82
6′	120.2	129.34	128.57	129.5	129.50	128.38
			6-*C*-β-Glucopyranoside			
1″	73.5	73.50	71.27	73.6	73.46	71.28
						71.03
2″	82.1	81.85	81.06	77.7	82.30	81.70
						80.41
3″	79.9	79.89	78.44	81.5	80.21	78.74
						78.65
4″	71.7	71.57	70.44	71.9	71.40	70.46
						70.13
5″	82.6	82.49	81.74	82.7	82.59	81.70
6″	62.9	62.87	61.52	62.8	62.83	61.38
	2-*O*-β-Xylopyranosyl			2-*O*-α-rha	2-*O*-β-glc	2-*O*-β-glc
1‴	106.8	106.76	105.98	102.6	106.40	105.66
			106.12			105.35
2‴	75.6	75.55	74.27	72.3	75.61	74.49
3‴	77.7	77.54	76.40	72.1	78.02	76.46
4‴	71.0	70.87	69.41	73.7	70.40	68.98
5‴	66.9	66.80	65.79	69.9	74.88	73.42
6‴				17.5	63.70	62.43
					6-*O*-(*E*)-caf	6-*O*-(*E*)-cou
1⁗					127.54	125.03
2⁗					123.15	130.11
3⁗					146.61	115.72
4⁗					149.50	159.78
5⁗					116.30	115.72
6⁗					115.00	130.11
α					114.35	113.76
β					146.78	144.30
C═O					168.91	166.31
						166.25

[a] Two chemical shift values are given for rotameric conformers. Abbreviations: nd = not detected, rha = rhamnopyranosyl, glc = glucopyranosyl, caf = caffeoyl, cou = coumaroyl. [b] In CD_3OD. [c] Chemical shifts from the 2D HSQC and HMBC spectra. [d] In DMSO-d_6.

respectively. The C−C linkage of the 6-*C*-glucosyl was confirmed by the HMBC cross-peaks at δ 6.48/73.4 (H-8/C-1″) and δ 5.05/165.2 (H-1″/C-7). The downfield shift of H-2″ (δ 4.38) confirmed the linkage between the *C*-glucosyl and the terminal glucose unit to be at the 2″-hydroxyl. The downfield shift of C-6‴ (δ 63.70) and H-6A‴ (δ 4.23) indicated the linkage between the caffeoyl moiety and the terminal glucose unit to be at the 6‴-hydroxyl. The cross-peak at δ 4.23/168.9 (H-6A‴/C═O) in the HMBC spectrum of **4** confirmed this substitution pattern. The molecular ion in the high-resolution ESI-MS spectrum of **4** was in accordance with the molecular formula $C_{36}H_{37}O_{18}$, corresponding to apigenin caffeoyldiglucoside. Thus, **4** was identified as the novel compound apigenin 6-*C*-(2″-*O*-(6‴-(*E*)-caffeoylglucoside)-β-glucopyranoside).

On the basis of the spectroscopic data, compounds **1**, **2**, **3**, and **5** were identified as luteolin 6-*C*-(2″-*O*-β-xylopyranosyl-β-glucopyranoside), apigenin 6-*C*-(2″-*O*-α-rhamnopyranosyl-β-glucopyranoside), apigenin 6-*C*-(2″-*O*-β-xylopyranosyl-β-glucopyranoside), and apigenin 6-*C*-(2″-*O*-(6‴-(*E*)-*p*-coumaroyl-glucoside)-β-glucopyranoside), respectively. The latter compound, which is one of the main flavonoids in the leaves of *O. triangularis* (**Figure 1**) has previously only been isolated from the leaves of cucumber (*Cucumis sativus*) and exclusively in plants that were treated with silicon and infected with *Sphaerotheca fuliginea* (*17*).

Signal duplication in the NMR spectra due to the presence of rotameric conformers, created by rotational hindrance at the $C(sp^3)-C(sp^2)$ glucosyl−flavone linkage in *C*-glucosyl-substituted flavones, was detected for apigenin 6-*C*-(2″-*O*-(6‴-(*E*)-caffeoyl-*O*-β-glucopyranosyl)-β-glucopyranoside) (**4**), apigenin 6-*C*-(2″-*O*-(6‴-(*E*)-*p*-coumaroyl-*O*-β-glucopyranosyl)-β-glucopyranoside) (**5**), apigenin 6-*C*-(2″-*O*-β-xylopyranosyl-β-glucopyranoside) (**3**), and apigenin 6-*C*-(2″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**2**). Rotameric conformers of flavone 6-*C*-glycosides (*18*−*22*) as well as flavone 8-*C*-glycosides (*23*) and proanthocyanidins (*24*) have previously been observed. Rotameric conformers of apigenin 6-*C*-(2″-(6‴-*O*-(*E*)-*p*-coumaroyl-*O*-β-glucopyranosyl)-β-glucopyranoside) (**5**) have not previously been reported. On the basis of the integration data from the 1D ^{1}H NMR spectra, the ratios of the major and minor conformers of **4** and **5** were determined to be 1:0.4 and 1:1, respectively.

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